The Vitamin D – Fibroblast Growth Factor 23 – Klotho Axis and Progression of Chronic Kidney Disease

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Chapter 7

Discussion, Conclusions and Final Remarks

7.1 General Discussion and Conclusions

Progression of CKD is closely linked to disturbances in calcium and phosphorus metabolism, some of which become apparent at a relatively early stage. These disturbances are for an important part driven by deregulations in the vitamin D-fibroblast growth factor (FGF) 23-Klotho axis. According to the current paradigm one of the earliest changes as kidney function starts to decline, is the rise in FGF23 levels. This most probably represents an adaptive mechanism to maintain normal phosphorus balance at the face of failing kidney function but at the expense of lower vitamin D synthesis. As renal function declines, vitamin D deficiency, phosphorus retention and FGF23 deregulation, resistance to the actions of PTH and vitamin D, and decreased presence and/or activation of several related receptors together result in excessive synthesis and secretion of PTH, leading to the development of secondary hyperparathyroidism (SHPT) and different forms of renal osteodystrophy. However, the effects of derangements in the vitamin D – FGF23 – Klotho axis extend beyond mineral metabolism. There is a considerable body of evidence showing reno- and vasculoprotective effects of vitamin D and Klotho and potentially harmful effects of excess FGF23 – making them interesting candidate targets for therapy. Interestingly, recent studies suggest crosstalk between the renin-angiotensin-aldosterone system (RAAS), a hormone system predominantly involved in the regulation of sodium and volume homeostasis, and the vitamin D – FGF23 – klotho axis[1]. This is of particular clinical relevance given the fact that the cornerstone treatment for CKD consists of pharmacological and non-pharmacological (e.g. dietary) interventions directed against deregulations in the RAAS. The studies described in this thesis aimed to provide insight in the role of the vitamin D – FGF23 – klotho axis in the development of CKD and their interaction with RAAS-blockade-based therapies.

The discovery of vitamin D and the elimination of rickets as a major medical problem represent one of the most important achievements in medicine over the past century. In the subsequent decades, the production, metabolism, and mechanisms of actions of vitamin D have been elucidated. Vitamin D acts via its receptor (VDR) to regulate calcium metabolism by stimulating its intestinal absorption, mobilization of calcium from the bone and renal reabsorption of calcium (together with PTH). One of the most important findings upon discovery of the VDR was that it was not only expressed in cells involved in calcium metabolism but also in a variety of other cells. This led to the discovery of functions of vitamin D not previously appreciated. Vitamin D, acting through VDR, exerts multiple effects in different systems such as the immune system, the central nervous system, and in renal and cardiovascular system.

In **Chapter 2** of this thesis we gave an overview of the renoprotective effects of vitamin D and VDR analogs (VDRA) and discussed the potential of VDRA to optimize renal protective strategies. In animal models, VDRA have been shown to reduce proteinuria, inflammation and fibrosis[2-4]. In addition, in several experimental models of CKD, VDRA have been shown to reduce residual proteinuria and tubulo-interstitial damage when given on top of RAAS blockade[5, 6]. Similar results were found in small-to-medium sized clinical trials in CKD patients with residual albuminuria despite RAAS-blockade[7]. These effects are achieved via direct protection of podocytes (either by inhibition of apoptosis or promotion of differentiation)[8] or via downregulation of pro-inflammatory or pro-fibrotic pathways (such as NF-kB, TGF-β)[3,9]. Importantly, VDRA may also act as negative regu-

lators of the RAAS by inhibition of renin transcription[10]. In fact, over the years, negative regulation of the RAAS became acknowledged as maybe the most important mechanism of renoprotection by VDRA. However, this may not be invariably so and may depend on the experimental model, as well as the type and the dose of VDRA used. For example, a recent preclinical study suggested that unlike paricalcitol, maxicalcitol prevented the development of interstitial fibrosis by blocking auto-induction of TGF-β resulting in inhibition of the pathway[11]. Importantly, this effect was shown to be RAAS-independent. In line with these observations, two synthetic vitamin D analogs prevented kidney fibrosis by specific inhibition of TGF-beta without affecting "classical" VDR responsive genes[12]. Importantly, both active vitamin D and paricalcitol also exerted antifibrotic effects but at the expense of hypercalcemia, which may promote vascular calcification on the long term, and hence limit therapeutic benefits.

What are the practical implications of these findings? Although inhibition of the RAAS might be an important determinant of the renoprotective effects of VDRAs, they cannot be considered equal to conventional RAAS blockers but more as adjunct to RAAS based therapy to down titrate proteinuria and downstream interstitial damage. This is of particular interest since interstitial damage can go largely unnoticed due to the lack of a validated biomarker allowing timely detection and therapy guidance. The development of new VDRAs with higher "antifibrotic" and less "calcemic" effects is an exciting option to further optimize renoprotective strategies. However, given the fact that the VDR is ubiquitously expressed, the question remains if the use of the new analogs would take away not only hypercalcemia but also other beneficial effects stemming from the classical activation of VDR. A head to head comparison in both experimental and clinical setting is certainly needed to answer this. In conclusion, VDRAs have the potential to become a clinically meaningful add-on strategy, continuing where RAAS-mediated renoprotection ends.

Reduction of blood pressure and proteinuria by RAAS-blockade has been the cornerstone of renoprotective treatment for patients with CKD for decades. However, despite its proven benefits, protection against adverse renal and cardiovascular events is far from complete. The high residual risk for these events could be related to insufficient RAAS-blockade (by inadequate dosing, limited tissue penetrance, or compensatory feedback mechanisms) or to the involvement of signalling pathways not affected by RAAS blockade. Combinations of RAAS blocking agents have been tested in different CKD conditions, providing further reductions in blood pressure and proteinuria, however, at the expense of higher incidence of adverse events and no obvious long term benefits. On the other hand, control of volume status is a relatively straightforward but often neglected therapeutic opportunity. Volume excess is consistently associated with a blunted response to RAAS blockade and, conversely, volume intervention by diuretic therapy and/or dietary sodium restriction restores and even enhances the effects of RAAS-blockade[13-16].

In **Chapter 3** of this thesis we investigated if the effects of RAAS blockade in combination with dietary sodium restriction can be further potentiated by addition of the VDRA paricalcitol in an experimental model of proteinuria-induced renal damage. Six weeks after the induction of adriamycin nephrosis, at established proteinuria, animals were treated with paricalcitol, lisinopril, the combination, or vehicle. Treatments were given during either a high or low-sodium diet for 6 weeks. Both paricalcitol and lisinopril individually and in combination, reduced proteinuria and glomerular and interstitial

inflammation during a low-sodium diet, but not during a high-sodium diet. All interventions also reduced focal glomerulosclerosis and interstitial expression of α-SMA during the low-sodium diet. The renoprotective effects of paricalcitol were not accompanied by blood pressure reduction. Results of the study described in this chapter are in line with previous studies showing additive effects of VDRA given in combination with RAAS blockers, as summarized in chapter 2. We extend these findings by showing that the combination of RAAS-blockade, dietary sodium restriction and VDRA may be a promising intervention to further retard renal function loss in CKD. In a recent clinical trial, we found that the combination of sodium restriction and VDRA treatment provided the strongest reduction of albuminuria, as compared with each individual treatment, during background RAAS-blockade. Thus, the renoprotective effects of VDRA treatment may depend on sodium status, which is in line with the interaction between sodium status and antiproteinuric effects for other classes of drugs[17]. It would be interesting to see if the observed sodium dependency of VDRA is specific for paricalcitol per se, or it may be a general feature of vitamin D analogs.

Besides proteinuria, which has been recognized as an independent and essential target for renoprotection, the extent of tubulo-interstitial damage has also been shown to predict response to RAAS blockade based therapies and subsequent renal function decline. The identification non-invasive markers other than proteinuria reflecting the intrarenal pathways of damage that may be utilized to monitor therapy response but also the factors that may modify response to RAAS blockade is of high importance, as it would potentially lead towards designing of more efficient treatment regimens.

In **Chapter 4**, we investigated the value of urinary vitamin D binding protein (uVDBP) as a marker of tubulointerstitial inflammation and fibrosis in a model of adriamycin nephrosis. In this study we tested whether uVDBP parallels renal damage and responds to therapy intensification in humans. In both settings, uVDBP was associated with markers of fibrosis and inflammation independently of albuminuria. The association with interstitial inflammation renders uVDBP an even more interesting candidate biomarker. In the experimental model, uVDBP was associated with markers of both early and late interstitial fibrosis (namely α-SMA and collagen III). However, uVDBP was increased early during progression of the disease before the onset of inflammation and fibrosis indicating that uVDBP can be used as a marker of early interstitial damage. Future studies should address whether uVDBP has predictive value for the progression of renal function loss and whether is a more suitable early marker of tubulointerstitial damage then the ones currently available.

In patients, uVDBP responded to intensified antiproteinuric therapy. However, uVDBP remained considerably higher than in healthy controls, indicating persistent tubulointerstitial damage in these patients. These findings are in line with preclinical studies showing dissociation of proteinuria and interstitial damage even when RAAS-blockade is optimized to achieve maximal reduction in proteinuria[18,19].

One reason for the incomplete therapeutic efficacy of RAAS-blockade could be that deregulated phosphate metabolism, arising from the disturbed vitamin D-FGF23-Klotho axis, adversely modulates RAAS-blockade efficacy. Higher phosphate levels are associated with worse disease outcome in proteinuric patients on RAAS blockade[20]. Recently, our group reported an association between higher levels of FGF-23 and reduced response to intensified RAAS blockade[21]. Whether FGF23 directly modulates the pharmacological

effects of RAAS blockade or whether is rather inducing renal damage which in turn contributes to therapy resistance[22] is unclear. Although a deleterious interaction between the RAAS and FGF23/Klotho system has been suggested, this had so far not been prospectively investigated.

In **Chapter 5** we investigated the renal effects of a high systemic FGF23 level, induced by exogenous (recombinant) FGF23 infusion, and its interaction with pharmacological blockade of the RAAS. We addressed this in the mouse model of unilateral ureteral obstruction, characterized by renal fibrosis, susceptible to antifibrotic effects of RAAS-blockade. This approach enabled us to directly assess possible interactions between FGF23 and RAAS-blockade efficacy without concomitant changes in mineral metabolism.

We showed that the pharmacological response to RAAS blockade might be influenced by FGF23. In our model, losartan induced upregulation of several RAAS related genes in the unaffected kidneys as a feedback response to the blockade of the angiotensin II receptor. In line with previous studies, Klotho expression was also induced by losartan[23]. These effects were reversed by FGF23 treatment, suggesting interference with the physiological effects of angiotensin II receptor blockade. The observed impaired upregulation of ACE2 gene, considered to be reno- and cardio-protective, in response to RAAS blockade may contribute to therapy resistance and to adverse (cardio)renal outcomes during high FGF23 conditions. The interaction between FGF-23 and RAAS blockade was further explored in the obstructed kidneys, where renal interstitial damage was extensive, including severe loss of Klotho expression. In this setting FGF23 did not have any direct effect on parameters of renal damage and showed only mild modulation of RAAS-blockade efficacy, apparent from small effects on renal expression of inflammatory markers. Clearly, the interaction between FGF23 and RAAS blockade was less apparent in the setting of severe renal damage, as characteristic of the UUO model, and we speculate that very low Klotho expression in the obstructed kidneys contributed to this discrepancy. The interaction between FGF23 and RAAS blockade deserves further investigation in other, preferably chronic models of CKD (i.e. proteinuric or subtotal nephrectomy). The use of these models would allow us to gain better insight into renal and systemic consequences of the interaction between FGF23 and RAAS blockade and the impact of Klotho as a potential modulator of the outcome. Surprisingly our results suggest that in the damaged kidney downregulation of Klotho would provide protection against impediment of RAAS blockade efficacy by FGF23. On the other hand, there is a substantial body of evidence showing that upregulation of Klotho provides not only renal but importantly also cardiovascular benefits in CKD[24, 25].

Cardiovascular morbidity and mortality are common in CKD. The high cardiovascular risk in CKD is at least in part driven by a progressive tendency to develop vascular calcification. The prevalence of vascular calcification is associated with many adverse clinical outcomes including ischemic cardiac events and all-cause and cardiovascular mortality. Hence, preventing or reversing vascular calcification may result in increased survival in patients with CKD. Both elevated serum FGF23 and low Klotho levels have been associated with increased vascular calcification in CKD and experimental studies have shown suppressive influence of soluble Klotho on vascular calcification [26,27]. However there is still controversy whether FGF23 directly contributes to vascular calcification and whether vascular tissue is directly responsive to FGF23 actions[28-34]. Although Klotho-independent actions of FGF23 have been shown, [35, 36] it is still widely accepted that the majority of

FGF23 effects is determined by the presence of Klotho in the target tissues. An interesting hypothesis has been suggested recently providing a mechanistic link between low Klotho and high FGF23 levels in the development of vascular calcification[30]. In this view, Klotho is locally expressed in vascular smooth muscle cells of human arteries and protects against vascular calcification by mediating FGF23 inhibitory effects on matrix mineralization. However, other studies investigating vascular Klotho expression yielded conflicting results arguing against direct role of FGF23 in vascular wall[33, 34]. Therefore, in **Chapter 6** we sought to determine the presence of the membrane-bound form of Klotho in the human arterial wall. Through a series of experiments we demonstrated the absence of Klotho on protein and gene expression level in human tissue. Moreover, upon treatment with recombinant FGF23, we detected an upregulation of Egr1 mRNA in mouse kidneys indicative of activation of the pathway. At the same time, the upregulation was absent in the aortas of FGF23 treated mice. Our findings argue against the presence of Klotho as a component of classical FGF23 signalling in the vessel wall and hence the presence and contribution of canonical FGF23/Klotho signalling in the vessels. If confirmed, our findings have important implications. They oppose the possibility of an active (direct) role for FGF23 in vascular calcification. In that respect FGF23 protrays vascular pathology associated with disturbed mineral metabolism and may not be the primary target to reduce vascular damage as demonstrated by a recent studies[37]. Second, in the absence of vascular expression of full length Klotho, vasculoprotective effects of Klotho are most probably mediated via its circulating soluble form, implying dependence on a distant source of Klotho, which is likely to be the kidney. Thus, with progression of CKD, the renal Klotho source gets exhausted, promoting progressive vascular disease. Interventions to up-regulate Klotho expression in the kidney could therefore be a promising treatment option. As we have shown in chapter 5, RAAS blockade results in upregulation of renal Klotho expression. However, in the same chapter we also show that high FGF23 levels may limit the efficacy of RAAS blockade and its ability to increase renal Klotho expression. The cardiovascular effects resulting from the restoration of renal Klotho expression by means of RAAS blockade in different FGF23 backgrounds would be interesting to investigate in the future studies.

7.2 Final remarks

Studies presented in this thesis provided more insight in the role of the vitamin D-FGF23-Klotho axis in the development of renal disease and its related cardiovascular complications, as well as its interaction with RAAS-blockade-based therapies. Our studies also represent experimental evidence that might be used to guide the development of new treatment options. Our data do not refute that RAAS blockade therapy is and will most probably remain the first-line treatment option for (proteinuric) kidney disease for the time to come, and every effort should be made to optimize its efficacy further. Disturbances in the vitamin D/FGF23/Klotho axis exert multiple effects extending beyond its role in mineral metabolism, making them interesting targets to further optimize renoprotective therapies and prevent CV complications. In that respect, the use of currently available VDRA is certainly an attractive option, along with the development of a new generation of VDRA with an even less calcemic and more “anti-fibrotic” profile. Corrections of possi-

ble adverse effects of FGF23 excess and detrimental effects of Klotho deficiency by means of hormone blockade or replacement (with blocking antibodies and recombinant protein treatments) are also attractive possibilities. In the meantime, more attention should be directed to the fact that these disturbances may be prevented at least in part by lifestyle changes including lower dietary sodium and phosphate intake.

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